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FINAL REPORT

BIOPROCESSING FEASIBILITY ANALYSIS

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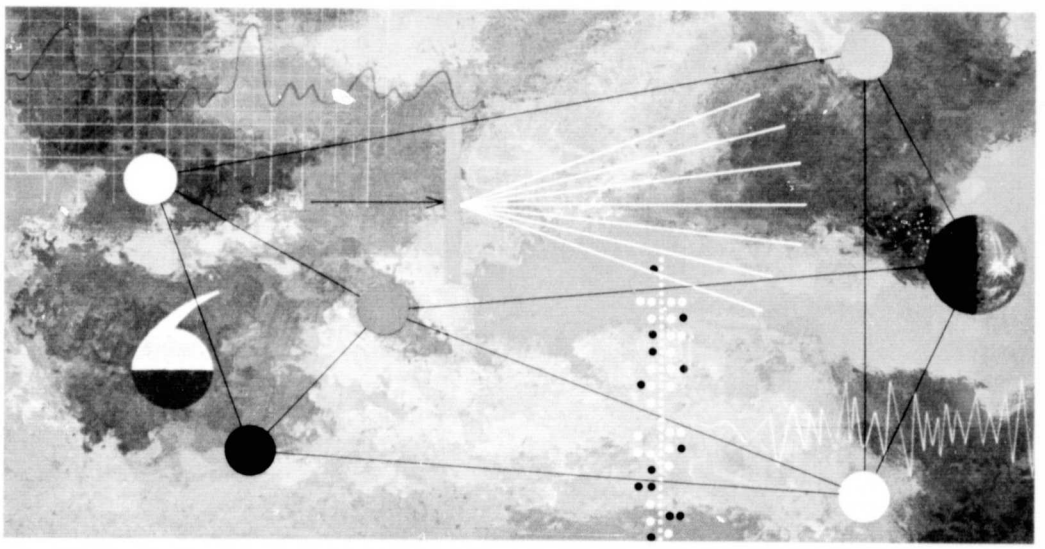

"Measurement is the basis of all knowledge"

Lord Kelvin

Beckman®

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Contract No. NAS9-15438

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National Aeronautics and Space Administration
Lyndon B. Johnson Space Center
Houston, Texas 77058

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SUMMARY

Whether or not the environment of space can be economically exploited for the preparation of unique biological products has been a controversial subject. The controversy stems from a lack of evidence showing that any specific product cannot be manufactured on earth but can probably be produced in the microgravity of space. This lack of evidence to support the claim and conjecture of aerospace-oriented investigators has evoked the criticism of the scientific community--perhaps rightly so.

Recognizing this deficiency, Beckman Instruments, Inc., in collaboration with scientists in appropriate disciplines, has defined an application of intrinsic economic value. An experimental program has been designed to develop the data required to support a decision to proceed with a space program.

The experimental program may provide a solution to the application problem on earth, or may indicate that solution requires recourse to the microgravity of space where advantages in separation resolution and throughput can be realized. *In either case, the initial ground-based research will be of unusually high intrinsic scientific worth.*

The application selected deals with the assay of a hormone produced by the thymus gland; it has immediate clinical relevance. The principal of the assay is straightforward and readily adapted to routine use. The critical limitation is the availability of indicator cells upon which the assay depends. Germ-free conditions for nude mice are currently required to provide a source of such indicator cells, thus severely limiting the availability of this assay. The object of this effort is to remove this restriction and make suitable cells widely available through the application of cell separation technology. Space may or may not be beneficial.

The ground-based research will be conducted at three centers to maximally utilize specialized scientific talents and facilities. Eighteen months will be required to accomplish the baseline investigations leading to spaceflight justification without compromise of scientific objectivity.

1.0 INTRODUCTION

Whether the microgravity of space can be economically employed for the preparation of biological materials has been actively debated for the past several years. That it has been subject to debate is due, in large part, to a dearth of empirically derived data from specific application studies which would show conclusively that space could be used effectively for a new or improved product. In addition to a lack of process feasibility information, the second factor in the determination of economic worth has been lacking, i.e., a commitment on the part of a manufacturing/marketing organization to finance a development program, to undertake the extensive product investigation effort required for a product to be employed *in vivo* to gain governmental approval, and then distribute the product.

This reluctance on the part of commercially-oriented organizations to actively participate in space investigations is not unusual. The risk is high, since feasibility has never been adequately demonstrated, and payoff is uncertain. The concept of opportunity cost is very germane in their considerations--especially when allocating the efforts of their innovative people. It is far more responsible to allocate resources in areas where payoff is more certain in a relatively short period of time.

It is reasonably apparent that NASA will need to conduct a feasibility demonstration program before widespread commercial interest can be attracted. Further, this program must survive both prospective and retrospective scientific and engineering scrutiny if data and claims resulting from it are to have any validity in the eyes of the scientific and commercial communities. To survive this scrutiny, the feasibility demonstration program must satisfy several criteria. These include:

- The application must be real, i.e., demand for the product must be apparent.

- The economic and technical advantages of going to space should be empirically demonstrable.
- *Alternative, ground-based approaches should be investigated and dismissed if found wanting. If ground-based techniques can be employed to obtain the desired end-product, the need for space will not have been shown and the feasibility demonstration program should be stopped.*
- The application selected for the feasibility demonstration program should be generally useful as a model system; i.e., extrapolation to other applications should be straightforward.
- The application should be independent of hardware. Checks and balances should be built into the program to avoid any implication of parochial interest.

Beckman has developed such a feasibility demonstration program in collaboration with eminent scientists in appropriate disciplines. The program is discussed in detail in the body of this report.

2.0 TECHNICAL DISCUSSION

2.1 Objective of the Program

The objective of the feasibility demonstration program is to make the bioassay for thymic hormone--a hormone of the thymus gland--widely available. The bioassay requires the use of indicator cells. The cells currently used must be harvested from mice raised in a germ-free environment, which makes widespread use of the assay unfeasible. We will attempt to provide an alternate source of these cells from conventional mice. Space *may* be useful in the development of this source.

2.2 Biology and Pathophysiology of the Thymus Gland

Both experimental and clinical experience indicate the importance of the thymus to immunologic competence. Nude mice with congenital absence of thymic tissue undergo wasting, are susceptible to infections, and die prematurely (Pantelouris, *Nature*, 217:370, 1968; Wortes, *Clin Exp Immunol*, 8:305, 1971). Humans with the DiGeorge syndrome, i.e., congenital absence or dysplasia of the thymus, also die from infection during infancy (DiGeorge, *Birth Defects*, 4:116, 1968; Kretchmer *et al.*, *New England J. Med.*, 279:1295, 1968) unless their thymus deficiency is corrected (Cleveland, *et al.*, *Lancet*, 2:1211, 1968). There is experimental evidence that stress from the environment, such as infection, contributes to the immunodeficiency that follows neonatal thymectomy (Bealmear and Wilson, *Cancer Res.*, 27:358, 1967).

The role of the thymus may extend beyond immunologic maturation to the maintainance of immune competence throughout life. For example, Kappler, *et al.* (*J. Immunol.*, 113:27, 1974) have shown in mice that effector, helper, and suppressor T cell function declines following adult thymectomy. Curtailment of thymic hormone secretion with age-related thymic involution (Bach, J. F., *et al.*, *Ann. NY Acad. Sci.*, 249:186, 1975) is probably a major factor in the waning of immunological vigor which occurs with advancing age (Makinodan *et al.*, *Adv.*

Geront. Res. e:171, 1972; Mackay, *Gerontologia* 18:235, 1972; Goidl *et al., J. Exp. Med.* 144:1037, 1976) and is an important determinant in longevity (Roberts-Thompson *et al., Lancet* 2:368, 1974). Premature thymic involution seems to contribute significantly to the pathogenesis of autoimmune disease in inbred New Zealand mice (Dauphinee and Talal, *J. Immunol.* 114:1713, 1973). Thymic hormone secretion may also be deficient in patients with systemic lupus erythematosis (Bach *et al., Ann. NY Acad. Asi.* 249:186, 1975) and perhaps with other autoimmune disorders as well.

A high percentage of thymomas are malignant and cause secondary metastases with secretory potential following thymectomy (Lahes, *Cancer* 15:224, 1962). Thymoma is associated with other disease states including acquired hypogammaglobulinemia (Siegal *et al., Birth Defects* 11:40, 1975), systemic lupus erythematosis (Goldstein and Mackay, *Brit. Med. J.* 2:475, 1967), bone marrow hypoplasia (Roland, *Am. J. Med. Sci.* 247:719, 1967) and chronic mucocutaneous candidiasis (Twomey *et al., J. Lab. Clin. Med.* 85:968, 1975). Thymitis and thymoma are also frequency concomitants with myasthenia gravis (Goldstein, *Lancet* 2:256, 1975).

It is, therefore, apparent that thymic hormone(s) has an important role in immunologic maturation, immunologic maintenance, and aging. Thymic abnormalities are concomitants of a number of disease states and may contribute to their pathogenesis.

2.3 Clinical Application of Thymic Hormone Assay

Thymic hormone assays have obvious clinical relevance. Such assays are necessary to correlate the status of thymic function with immunodeficiency and to establish the apparent relationship between thymus dysfunction and the pathogenesis of certain diseases.

A summary of immediate and potential diagnostic applications of the assays would include the following:

- Study of aging and abnormalities of aging.

- Identification of primary immunodeficient patients where thymic function is impaired (DiGeorge syndrome, Nezilof syndrome, Severe Combined Immunodeficiency Disease (SCID), and ataxia telangiectasia have been identified as such).
- Search for other disease states where thymic hormone secretion may be subnormal (e.g. cancer, lymphoma, leprosy, sarcoid, and systemic lupus erythematosis).
- Determination of the usefulness of the thymic hormone bioassay for detecting thymomas and for identifying thymomas which are malignant.
- Use of the thymic hormone bioassay to monitor patients with malignant thymomas after thymectomy for the appearance of secreting metastases.
- Determination of the effect of therapeutic mediastinal irradiation (as used for cancer) upon thymic hormone secretion.
- Use of the thymic hormone assay to determine the technical adequacy of thymectomy as used to treat myasthenia gravis, for example.
- Application of the assay clinically to the workup of patients with other diseases identified through the preceding studies where circulating thymic hormone levels are abnormal.

In addition to diagnostic applications, hormone measurements appear essential for the establishment of therapeutic schedules with various thymic preparations. A number of pharmaceutical companies have become interested in producing thymic hormone. Hoffman LaRoche is now extracting thymosin from bovine thymuses (Goldstein, A., *et al., Proc. Nat'l. Acad. Sci.* 56:1010, 1966). Miles Laboratories have also announced their interest in thymic hormone. Ortho Laboratories is involved in the synthesis of peptides with thymic hormone activity. The assay will therefore be required in the pharmaceutical industry to determine the activity of extracts and synthetic peptides, to establish the biologic half-life of preparations shown to have thymic hormone activity, and to standardize commercial products.

2.4 Thymic Hormone Assay Procedures

The following definitions are presented to aid the reader who may be unfamiliar with terms that will be employed in subsequent discussions:

Thymic hormone bioassay (Twomey). This assay calls for the measurement of Thy 1.2 antigen induction on genetically programmed null lymphocytes where nonspecific induction is inhibited with ubiquitin and raw data is corrected against a thymopoietin standard. This bioassay has been developed in one of our laboratories (Twomey, J.J., *et al., Proc. Nat'l. Acad. Sci.* 74:2541, 1977; Lewis, V., J.J. Twomey, *et al., Lancet II* 8036:471, 1977).

Indicator cell preparation. This is the cell preparation used to measure Thy 1.2 antigen induction on null lymphocytes. Heretofore, these cells have been obtained from the spleens of germ-free athymic mice housed in a special colony under strict germ-free conditions. Fresh cells, in the effluent from nylon column preincubations are employed. In the present system these include >80% lymphocytes, <1% Thy 1.2^+ and <5% membrane Ig^+ cells. They should be >90% viable by trypan blue exclusion. Thus, most of these cells are null lymphocytes. For reliable bioassay results (<6% variability) at least 30% of these cells must be susceptible to induction to express Thy 1.2 antigen during 18 hours of incubation at 37°C with 1 µg thymopoietin per 0.2 ml.

Null lymphocytes. These are morphologically mature lymphocytes that lack membrane markers for T cells, B cells, or K cells; do not adhere to foreign surfaces such as nylon; are nonphagocytic; and are deficient in cytoplasmic lysosomal enzymes (Stobo *et al., J. Exp. Med.* 138:71, 1973; Twomey *et al., Proc. Nat'l. Acad. Sci.* 74:2541, 1977). The nucleus has relatively mature chromatin although nucleoli may be observed. In germ-free nude mouse indicator cell preparations, we can induce 40-50% of the null cells in indicator cell preparations to express Thy 1.2 antigen under optimal stimulation. Null lymphocytes are to be distinguished from primitive precursor stem cells (Dickie *et al., Exp. Hemat.* 1:36, 1973) or lymphoblasts which can express Thy 1.2 antigen (Greaves, *Excerpta Medica* 3:39, 1974).

2.4.1 Assay by Erythrocyte Rosette Formation

Bach *et al.*, first developed a bioassay for T cell induction using the property of erythrocyte rosette formation (Proc. Leuk. Cult. Conf. 7:271, 1973). Pre-incubation with azathioprine lowers this rosette formation by a few percentage points. Bach uses the ability to reverse this inhibitory effect of azathioprine to measure thymic hormone. This assay has shortcomings. Since it depends on the accurate measurement of 1 to 3% changes in rosetting, the assay is subject to considerable error. It remains to be proven that induction reflects thymic hormone. The significance of rosette formation remains to be determined. The assay is of demonstrably lower sensitivity than the Twomey assay. Other laboratories have experienced great difficulty in getting the bioassay to work.

2.4.2 Assay of Cellular Cyclic AMP

Astaldi *et al.*, used the knowledge that thymic factors increase the content of mouse thymocyte cyclic AMP to develop another indirect bioassay (Kook and Trainin, *J. Exp. Med.* 133:193, 1974; Astaldi *et al.*, *Nature* 260:713, 1976; Astaldi *et al.*, *J. Immunol.* 119:1106, 1977). Obviously this parameter is affected by many factors other than thymic hormone.

2.4.3 Induction of Thy 1.2 Antigen

The bioassay developed by Twomey is a direct measurement of serum thymic hormone activity. It measures the induction of Thy 1.2 antigen on genetically programmed null lymphocytes from the spleens of germ-free nude mice (Twomey *et al.*, *Proc. Nat'l. Acad. Sci.* 74:2541, 1977; Lewis, Twomey, *et al.*, *Lancet II*(8036):471, 1977). Induction incubations are for 18 hours at 37°C. The presence of Thy 1.2 antigen on cell membranes is measured using specific antiserum, complement, and a sensitive enzymatic cytotoxicity test (Twomey, *et al.*, *Cancer*, In Press). Nonspecific induction is inhibited by the addition to induction incubations of 125 µg ubiquitin per 0.2 ml (Brand *et al.*, *Science* 193:319, 1976) which also increases the sensitivity of indicator cells to low concentrations of thymic hormone (Twomey *et al.*, *Proc. Nat'l. Acad. Sci.* 74:2541, 1977). The assay is dependent upon the fact that there is a direct relationship between thymic

hormone concentration and percent indicator cells induced to express Thy 1.2 antigen ($r = .997$, $p < .001$) (Lewis, Twomey, *et al.*, *Lancet II* 8036:471, 1977). Day-to-day variations are corrected against a known thymopoietin standard. Values in sera are expressed as equivalents to known amounts of thymopoietin. This bioassay can detect <0.2 ng thymopoietin per ml with excellent reproducibility.

Age-related values for immunologically healthy individuals have been determined to establish a normal range of serum thymic hormone activity (Lewis, Twomey, *et al.*, *J. Clin. Endocrin. Met.*, under review). Using cardiac surgery patients, serum thymic hormone activity has been correlated with the concomitant degree of thymic involution. A slight increase was recorded after the first age decade. Values began to decline after age 35 years. Activity dropped precipitously to negligible levels at about 60 years of age. It would appear that considerable thymic involution takes place before circulating thymic hormone activity declines. This suggests that the normal juvenile thymus has considerable functional reserve.

The fact that thymic deficiency can be established with this bioassay was proven with studies on patients with DiGeorge syndrome (Lewis, Twomey, *et al.*, *Lancet II* 8036:471, 1977). In studies on 15 patients with severe combined immunodeficiency, it was shown for the first time that this, too, is a severe thymic deficiency disorder. This provided new insight into the pathyphysiology of the disease and provided a rationale for its treatment with thymic replacement (Hong *et al.*, *Lancet* 2:1270, 1976). We also have evidence of functional thymic deficiency with ataxia telangiectasia, where the thymus is known to be dystrophic.

Bioassay determinations have been performed on 31 patients with myasthenia gravis. Up to the sixth age decade values fell within the normal range. Nine of ten patients over age 50 years had significantly elevated values. Negligible activity was found in serum from 19 patients one week following thymectomy. This suggests that elevated peripheral levels of thymic hormone is not essential to the pathophysiology of myasthenia gravis. Elevated levels

among older patients, in whom values normally decline, may be secondary to thymitis. However, thymic hormone may still have a role in the disease. For example, such patients may have increased sensitivity to an effect of thymic hormone at the neuromuscular junction. A possible cause for this could be autoantibody to acetylcholine receptors.

Thus, the bioassay has proven its clinical usefulness and is in increasing demand. The major limiting factor is the availability of indicator null lymphocytes from germ-free mice. Nude mice maintained in a conventional environment are unsuitable for these studies. Their spleens are small and contain an inadequate concentration of T cell precursors. This results in both unreliable and poorly reproducible bioassay results. Presumably, the stress from infection (endotoxin?) depletes the spleens of these mice of Thy 1.2^{+} precursor cells.

There are great logistic and economic limitations to a germ-free nude mouse colony. In fact, any such colony, even in most experienced hands, could become worthless at any time through inadvertant contamination. Germ-free housing is also needed for commercially available nude mice with "associate flora" of low virulence (Schoeffler flora) or for neonatally thymectomized, irradiated, and bone marrow-reconstituted conventional mice (Bealmear and Wilson, *Blood* 30:112, 1967).

An obvious potential source of indicator cells is a null cell line maintained in culture. Although this approach may appear promising on the surface, it suffers a number of pitfalls: 1) serum required in the culture medium must be free of thymic hormone, e.g., nu/nu, post-thymectomy or aged mouse serum; 2) serum required in the medium must be devoid of heteroantibody which would render the cell unuseable in subsequent bioassays. These serum-related problems are very restrictive. Since it is highly unlikely that the cells can be maintained in serum-free media, it is also highly unlikely that a cell line can be established and maintained.

Consideration was also given to the use of primitive human leukemic cells lacking T cell markers. This service was dismissed, however, for the following reasons:

- These cells represent heterogeneous cell population from patient-to-patient.
- An undetermined percentage of these leukemias may be uncommitted precursors of the myeloid or B cell series and not responsive to T cell indication.
- They are abnormal cells; in some or all instances they may be resistant to further differentiation.

3.0 PROGRAM PLAN

The initial phases of the program are devoted to ground-based research which will answer two questions:

1. Can null lymphocytes be isolated from a readily accessible source, such as C3H mouse spleens, with sufficient purity, yield, and functional capacity to be useful in the thymic hormone bioassay?
2. Can null lymphocytes be stored and transported in a state which will allow the recovery of functional cells?

Answers to the above questions are required to determine whether a microgravity environment might provide a sufficient increment in purity and throughput to make the space isolation of null cells feasible and economically justifiable.

Ground-based research will be conducted in two phases. During Phase I, characteristics of spleen cell subpopulations which might be exploited to achieve the required degree of separation will be explored. During Phase II, actual cell separation by one or more techniques identified during Phase I will be attempted.

3.1 Phase I

Exploration of separability of functional null cells for use in the thymic hormone bioassay.

3.1.1 Task 1--Storage and Shipment of Null Cells from Nu/Nu Mice

Central to any consideration of widespread use of null cells for the thymic hormone bioassay, especially from a single source, e.g., space, is a determination of whether the cells can be stored and transported while retaining their functional characteristics. Cells must survive at least one freeze/thaw cycle and possibly more if they are to be prepared and then shipped to various laboratories throughout the world.

Functional survival of the cells will be established according to the following protocol.

3.1.1.1 Collection of Cells

Nu/nu mice, 12-16 weeks of age, will be employed. The mice will be sacrificed by cervical dislocation. The abdomen will be opened using a flamed scissor and the spleen excised and placed in a petri dish containing ice-cold RPMI-1640 medium (GIBCO). Cells will be freed from the spleens by repetitive injections of medium using a syringe and needle. The medium containing the cells will then be transferred to a tube and allowed to stand at room temperature for 10 minutes to allow clumps to sediment. Cell preparations will be tested for viability by trypan blue exclusion. Only cell preparations with greater than 95% viability will be used for further testing.

3.1.1.2 Depletion of B Cells and Monocytes

The method of Trizio and Cudowicz (*J. Immunol.* 113:1093, 1974) will be employed. Briefly, 0.6 g nylon wool from Fenwal infusion sets is packed loosely into 12-ml plastic disposable syringes under sterile conditions. These columns are equilibrated in a tissue culture incubator at 37°C with medium 199 with 5% BSA. About 1.2×10^8 cells are then added to the columns and incubated for an additional 45 minutes. Non-adherent cells are then recovered in the effluent.

3.1.1.3 Storage of Cells

An aliquot of the cells obtained as described in paragraph 3.1.1.2 will be assayed for activity to provide pre-storage baseline data. Remaining cells will then be suspended in RPMI 1640 plus 10% DMSO and frozen in liquid nitrogen at 1°C per minute until -50°C is reached and then rapidly to -90°C at which temperature they will be stored until tested. Frozen cells will be shipped in liquid nitrogen in plastic screw cap Nunce (Denmark) vials. Prior to testing, the cells will be thawed rapidly in a warm water bath (37°C).

3.1.1.4 Test of Stored Cells

The test procedure will be the Twomey thymic hormone bioassay (Lewis, Twomey *et al., Lancet II*(8036):471, 1977). Induction incubations will include 0.5 x 10^6 test cells in 0.2-ml medium and 125 µg ubiquitin. In separate paired incubations the following stimuli will be added: (a) thymopoietin in 1 µg as a standard, (b) thymopoietin 0.6 ng, (c) thymopoietin 0.4 ng, (d) thymopoietin 0.2 ng, and (e) no thymopoietin. Optimal stimulation will be assessed with incubation (a) and should exceed 30% Thy 1.2^+ cells. The lesser concentrations of thymopoietin which will be compared with the dose response curves previously obtained using freshly prepared germ-free nude mouse spleen cells (Figure I, *Lancet II*(8036):471, 1977). The induction incubations will be for 18 hours. Cytotoxicity will be measured using the protease solubilization test (Stewart and Goldstein, *J. Lab. Clin. Med.* 84:425, 1974) since it is considerably more sensitive to minimal cell injury as occurs in this system than other procedures such as dye exclusion or isotope release (Twomey, *et al., Cancer,* In Press).

The information obtained from these tests will be of great value in assessing how to proceed to further tasks. Some attrition is acceptable because the assay is carried out on a small scale.

3.1.2 Task 2--Null Cell Characterization

During Task 2, spleen cells from C3H and nude mice will be characterized with respect to those properties which should be useful in enriching their null cell content. Parameters of interest will include cell density, size, electrophoretic mobility and surface properties as detected by partition in two-phase aqueous polymer systems.

The principal objective of this program is the isolation of reactive null cells from populations of spleen cells. Since the characteristic reactions of null cells with which we are concerned involve cell membranes, it seems likely that separation techniques based on membrane properties might have the highest probability of success. Such techniques are not in wide use, however, the most commonly applied cell separation procedures being based on centrifugation.

Centrifugation procedures separate on the basis of cell geometry and/or density. Since there would seem to be little reason to expect that lymphocyte subpopulations engaged in a particular immune function should exhibit a unique and uniform cell geometry or density, centrifugation techniques will probably not be specific enough to solve the present problem. On the other hand, there is some evidence which suggests that the density distribution of null cells may differ somewhat from that of T cells (Bach, M. *et al., Ann. NY Acad. Sci.* 249:316, 1975), so size and density distributions will be examined in our program.

At present there are three broad classes of cell separation procedures which select on the basis of surface properties: procedures which depend on an adherence reaction between cells and other cells and a surface or matrix (Shortman, K., *Ann. Rev. Biophys. Bioeng.* 1:93, 1972), preparative cell electrophoresis (Hannig, K., *Jahrbuch Max Planck Ges, 5,* 117, 1968), and countercurrent distribution of cells in two-phase aqueous polymer solutions (Albertson, P.-A in "Partition of Cell Particles and Macromolecules," 2nd Edition, Wiley-Interscience, New York, 1971; Walter, H., in "Methods in Cell Biology," Vol. 9, p. 25, D.M. Prescott (ed.), Academic Press, New York, 1975). We propose to utilize all three of these approaches in Phases I and II of this project, as well as other techniques which do not conveniently fall in the surface property classification.

The spleen cell surface properties we will characterize will be the cellular electrophoretic mobility distribution and the set of surface properties which determine cell partition behavior in two-phase aqueous polymer systems.

3.1.2.1 Electrophoretic Mobility

The electrophoretic mobility, u, is defined as the velocity, v, per unit field strength attained by a suspended cell exposed to a uniform electric field E. That is:

$$u = v/E \qquad (1)$$

The mobility is a direct measure of the net cell surface charge density, σ, since it is related to this parameter by:

$$u = \sigma/\kappa\eta \qquad (2)$$

where

η = viscosity of suspending medium

$\kappa = \left[\frac{8\Pi N_a e^2 I}{1{,}000\varepsilon KT}\right]^{1/2}$ is the Debye-Huckel parameter

N_a = Avogadro's number

ε = dielectric constant of suspending medium

K = Boltzmann's constant

T = absolute temperature

$I = 1/2 \sum_i c_i z_i^2$

c_i = molar concentration of i^{th} ionic species

z_i = valence of i^{th} ionic species

Since the surface charge density is determined by the number and distribution of the acidic and basic chemical groups present on the cell surface (sialic acid, acidic and basic amino acids, etc.) the electrophoretic mobility is directly proportional to a parameter which reflects the biochemical character of the cell coat. This parameter is of interest because there is a preparative electrophoretic procedure available, continuous particle electrophoresis (CPE), that separates subpopulations of cells on the basis of their mobilities which may well prove to be valuable in solving the present problem.

Electrophoretic cell separations are necessarily conducted in free liquids lacking any support media such as gels which would impede cell movement. In one approach, the liquid is stabilized by a density gradient. This may introduce difficulties such as osmotic damage or clumping due to the gradient additive, or interference between cell-density related and electrophoretic forces which may degrade the resolution. Electrophoresis of cells in a gradient-free

liquid column in microgravity has been tested (Barlow, G.H. *et al.*, in "Bioprocessing in Space," D. R. Morrison (ed.), Proceedings, 1976 NASA Colloquim, Houston, Mar 10-12, 1976, NASA Report TM X-58191, Jan, 1977); however, it proved difficult to control chamber wall zeta potential, and to introduce and remove sample material.

The CPE method (Hannig, K., *Jahrbuch Max Planck Ges., 5,* 117, 1968; Strickler, A. in "Separation Techniques in Chemistry and Biochemistry," R.A. Keller (ed.), Marcel Dekker, NY, 1967) avoids most of the problems of liquid columns. In this method, a lateral electric field is applied to a relatively thin buffer layer flowing continuously between a pair of parallel, non-conductive plates. The sample, entrained continuously into the buffer as a thin stream, is separated into a steady-state diverging fan of components of different electrophoretic mobility. The components are collected via an array of closely spaced ports at the end of the chamber into an antibiotic-rich medium.

A limitation of continuous electrophoresis in earth gravity is that its "figure of merit"--the extent to which it combines resolution with processing capacity--is relatively low. Theory suggests that performance in microgravity may possibly combine relatively high resolution with a manifold increase of processing rate (Strickler, A., AIAA 15th Aerospace Sciences Meeting, Los Angeles, Jan 24-26, 1977, paper No. 77-233, American Institute of Aeronautics and Astronautics, 1290 Avenue of the Americas, NY 10019).

The electrophoretic properties of lymphocytes, and the possibility of separating lymphocyte subgroups electrophoretically, have already attracted the attention of investigators. In a number of studies on lymphocytes of various mammals, including human and mouse lymphocytes (Wioland, M. *et al., Nature New Biology* 237, 274, June 28, 1972; Wiig, J.N. and S. Thunold, *Clin. Exp. Immunol. 15,* 497, 1973), using the microelectrophoresis technique, low and high mobilities could be associated respectively with properties of B and T lymphocytes. In other studies, (Nordling, S. *et al., Eur. J. Immunol.* 2:405, 1972; Zeiller, K. and G. Pascher, *Eur. J. Immunol.* 3:614, 1973) lymphocytes were obtained from various organs and separated preparatively by continuous flow electrophoresis.

The investigations uniformly showed properties in the lower-mobility portion of the spectrum associated with B cell function, and T cell-associated properties in higher-mobility fractions. A study by Boehmer (Boehmer, H.V., *J. Immunol.* 112:70, 1974), on spleen cells from CBA H. Weil and BALB/c mice fractionated by continuous electrophoresis identified a low mobility B cell fraction by immunofluorescence test, a high mobility T cell fraction identified by the presence of theta antigen, and an intermediate-mobility "double negative" cell fraction showing neither B nor T identity.

In order to determine if there is any point in applying CPE to the null separation problem, information regarding the electrophoretic properties of null cells relative to the cells with which they coexist in the spleen must be obtained. Neither C3H nor nude mice have been examined for the electrophoretic characteristics of their spleen cell populations. Such data may be obtained by performing analytical cell electrophoresis on the relevant cell populations.

Analytical cell electrophoresis allows the electrophoretic mobility of individual cells to be measured. The cells are observed microscopically and their individual velocities measured by timing their migration over a fixed distance after application of a known electric field. Analytical cell electrophoresis does not produce a physical separation of electrophoretically distinct subpopulations but the mobility data it provides can be accumulated into a frequency histogram which represents the mobility distribution of the cell suspension under observation. These distributions can be used to predict the electrophoretic separability of the various subpopulations present. It is this type of information which will be obtained during the electrophoretic characterization of the spleen cell populations of interest in the present study.

3.1.2.2 Cell Partition Behavior

The second set of surface properties we will investigate in the mouse spleen cell populations will be those which determine the partition behavior of cells in two-phase aqueous polymer systems. When aqueous solutions of two different polymers are mixed above certain concentrations they frequently form immiscible, liquid, two-phase solutions. Each of these phases contains predominantly one

of the polymer species but consists of more than 90 percent water and can be buffered and made isotonic by the addition of low molecular weight species. If a cell or particle suspension is added to such a system, then shaken, the cells--upon re-equilibration--are frequently found to have partitioned unequally between one of the phases and the interface. This preferential partition behavior can be used as the basis of a separation procedure for differing cell populations since partition in these systems is determined directly by cell membrane properties (Walter, H. and E.J. Krob, *FEBS Letters* 78:105, 1977, and Walter, H., in "Methods of cell separation," Vol. 1, p. 307, N. Catsimpoolas (ed.,), Plenum Press, NY, 1977).

Cell populations which have related, but not identical surface properties seldom exhibit sufficiently different partition behavior to be separated in a single extraction. In such cases, multiple partitions are carried out via countercurrent distribution (CCD) to effect the separation. CCD in phase systems derived from dextran/polyethylene glycol (PEG) mixtures has proven to be an extremely sensitive and valuable preparation technique in cell biology; a wide variety of cell types can be subfractionated by this technique (Walter, H., in "Methods of cell separation," Vol. 1, p. 307, N. Catsimpoolas (ed.), Plenum Press, NY, 1977). Human and rat red cells partition very differently from their respective leukocyte fractions, for instance, and can be easily separated via CCD (Walter, H., E. J. Krob, R. Garza, and G.S. Ascher, *Exp. Cell Res.* 55:57, 1969). The leukocyte fractions from various mammalian species have in turn been fractionated this way (Walter, H., E.J. Krob, and G.S. Ascher, *Exp. Cell Res.* 55:279, 1969; and Walter, H. and H. Nagaya, *Cell Immunol.* 19:158, 1975), as has a mouse leukemic cell line (Gersten, D.M. and H.B. Bosmann, *Exp. Cell Res.* 87:73, 1974, and Gersten, D.M. and H.B. Bosmann, *Exp. Cell Res.* 88:225, 1974) and, in work done before T and B cells were recognized, mouse spleen cells (Brunette, D.M. *et al., Cell Tissue Kinet* 1:319, 1968). It has been shown that stored human red cells have markedly different partition coefficients from fresh erythrocytes (Walter, H., R. Garza, and F.W. Selby, *Exp. Cell Res.* 49:679, 1968). There is also strongly suggestive evidence that human erythrocytes can be distributed on the basis of cell age by CCD (Walter, H. and F.W. Selby, *Biochem. Biophys. Acta* 112:146, 1966). Certainly in other systems the maturity of the

cell affects its partition behavior. Rapidly regenerating rat liver cells, for instance, have a higher partition coefficient than normal liver cells (Walter, H. *et al., Exp. Cell Res.* 82:15, 1973). Similarly, the relative position of rat epithelial cells in a CCD curve depends strongly on cell age and location in the epithelium (crypt or villus) (Walter, H. and E.J. Krob, *Exp. Cell Res.* 91:6, 1975). The dextran/PEG phase system used in the above work has therefore been amply proven as a separation medium.

It is clear that the success of CCD as a separation procedure depends primarily on the tendency for the cell species of interest to associate with one of the two phases, or their interface, more strongly than other cell types with which it is usually found. Based on the results of a variety of surface chemical, electrochemical, and analytical techniques we have developed a reasonable picture of the determining factors important in cell partition in these systems.

An approximate analytical expression for the cell partition coefficient which describes the tendency of a population of cells to accumulate in one of the two phases (or the interface) is given by:

$$K = \exp\ (1/kT)\ [Q_{eff}(\psi^{II}-\psi^{I}) + (\gamma^{II}-\gamma^{I})A] \qquad (3)$$

where

K = ratio of concentrations of partitioned material in Phases I and II, C^{I}/C^{II} (or between one phase and the interface)

Q_{eff} = effective cell surface charge

$(\psi^{II}-\psi^{I})$ = electrostatic potential difference between phases

$(\gamma^{II}-\gamma^{I})$ = difference in interfacial free energy of cell/polymer solution interface in two phases

A = cell surface area

This equation illustrates mathematically what has been shown in a general way experimentally, namely, that partition depends mainly on two cell properties, one of which is the surface charge and the other of which has to do with a

non-charge interaction between the membrane and the phases (the cell/phase interfacial free energy). It brings out the important point that the partition coefficient depends *exponentially* on the surface properties of interest, implying that small differences in surface charge or in the degree to which the phases interact with the membrane can cause large differences in partition behavior.

The equation also indicates that the surface charge is important in determining K only if an electrostatic potential difference exists between the phases. We have shown that this phase potential is produced by the unequal partition of certain salts, such as phosphates, in dextran/PEG phase systems (unpublished).

Only a few ions produce this effect, however, so it is possible to manipulate the magnitude of the potential difference, and hence the dependence of cell partition on surface charge, simply by manipulating the salt composition. Typically, the ratio of phosphate to chloride is varied at constant ionic strength, producing potentials from 0 to approximately 2 mV (top, PEG-rich phase positive) (Reitherman, R. *et al.*, *Biochim. Biophys. Acta* 297:193, 1973). This small change in phase potential is sufficient in many cases to increase partition into the top phase from zero to essentially 100% of the cells of a given type (Walter, H., E.J. Krob, and D.E. Brooks, *Biochemistry* 15:2959, 1976). By appropriate choice of salt content then, phase systems can be formed which will partition cells completely or partially on the basis of cell surface charge, or with no dependence on cell charge at all. This flexibility is of considerable importance for the problem at hand.

The expression for K indicates that charge is not its only determinant. The interfacial free energy of the cell/solution interface also can play a strong role. This free energy will be determined largely by the degree to which one or the other of the phase polymers adsorbs to the cell surface, thus lowering the free energy between the polymer-coated cell and the phase in which the polymer predominates. The competitive adsorption of the two polymer species depends in turn on the chemical nature of the polymers and on a variety of cell membrane properties. Few of these membrane properties have been

identified as yet, but in a PEG-dextran system having no phase potential difference there is good evidence that PEG-membrane interactions are stronger, and partition into the PEG-rich phase higher, the greater the ratio of polyunsaturated to mono-unsaturated fatty acids in the membrane lipid (Walter, H., E.J. Krob, and D.E. Brooks, *Biochemistry* 15:2959, 1976). This phase system, therefore, apparently detects differences in membrane properties associated with differences in lipid composition, possibly differences in packing of lipid molecules in the bilayer.

One way in which the interfacial free energy term can be made dominant for a particular subpopulation of cells is to chemically bond a specific affinity ligand to one of the two polymer species. Such a procedure greatly enhances the adsorption of the reacted polymer species to those cells which contain the binding site for the affinity ligand. With the appropriate choice of conditions this preferential adsorption will cause only the coated cells to be drawn into the phase in which the polymer-ligand species predominates, thus producing the specific separation desired (Flanagan, S.D. *et al.*, *Nature* 254:441, 1975; Walter, H. and E.J. Krob, *FEBS Letters* 61:290, 1976; and Eriksson, E. *et al.*, *Molecular and Cellular Biochem.* 10:123, 1976). Since the entire soluble phase acts as a support medium for the affinity ligand, the ligand has much greater access to the cell surface than it would if coupled to a sepharose or gelatin matrix. Affinity-based separations of cells should therefore be considerably more effective when partition rather than column chromatography is the basic procedure employed.

Because of the low concentrations of polymers employed (typically 4-5% w/w) and because a wide variety of metabolites, buffers, and support media can be incorporated into the phase systems, CCD is an extremely gentle technique. Cell viabilities remain high after separation (Brunette, D.M. *et al.*, *Cell Tissue Kinet* 1:319, 1968). In fact we have found that the polymers exert a protective action on the cells undergoing CCD, viabilities after several hours being higher in the phase systems than corresponding controls not exposed to polymers.

The strengths of the partition approach to cell separation, then, lie in its sensitivity to a variety of membrane characteristics, in the gentle nature of the procedure, and in the control which the experimenter has over the conditions which determine the partition coefficient. We think it is an appropriate and promising approach to the problem of separation of mouse spleen cell populations for the following reasons. Firstly, differences in the mean surface charge densities of mouse T, B, and null cells are suggested from the work of Boehmer (Boehmer, H.V., *J. Immunol.* 112:70, 1974). Since the partition coefficient in charge-dependent phase systems depends exponentially on surface charge density it should be possible to isolate--to an unknown degree--the subpopulations of interest. Secondly, we have examined human peripheral blood mononuclear cell distributions after CCD in phase systems bearing a lipid affinity ligand (a PEG-palmitic acid ester) and found large differences in B and T cell partition behaviour (unpublished). Moreover, the mean partition coefficients for null and T cells appeared to be quite different in this system. While it is recognized that there can be very significant differences in lymphocyte surface properties from human and rodent sources (Vassar, P.S., E.M. Levy, and D.E. Brooks, *Cell. Immunol.* 21:257, 1976) the above results nonetheless suggest that polymers bearing hydrophobic affinity ligands will be promising systems to apply to the null cell separation problem.

In order to examine directly the feasibility of obtaining null cell separations via CCD we propose to carry out a number of single-step partition experiments on the cell suspensions described below. These experiments will characterize the cells according to their mean partition coefficients in phase systems which separate on the basis of charge and/or on the basis of those membrane properties which determine the extent to which the two polymers comprising the phase system compete for adsorption sites on the cell surface. Knowledge of these partition coefficients will provide information on the separability of the cell populations involved and on the systems most likely to be successful in solving the problem at hand.

3.1.2.3 Characterization of C3H Spleen Cells After Passage Through a Nylon Wool Column

C3H spleen cells depleted of B cells will be characterized in order to determine the general range of the parameters of interest and to provide an analytical description of the cell population which will be subjected to our separation procedures. C3H spleen cells will be frozen in Houston according to the protocol established in Task 1 and shipped to Vancouver in order to control against any freezing/storing/thawing effects in subsequent experiments. They will then be revived by the established procedure and incubated on nylon wool columns (Fenwall) for 45 minutes as described by Trizio and Cudowicz (Trizio, D. and J. Cudkowicz, *J. Immunol.* 113:1093, 1974). The effluent cell population, which consists of T and null cells, will then be characterized as follows.

3.1.2.3.1 Cell Size Distribution

The apparent volume distribution of the population will be determined using a Particle Data Model 112 CLTH/TWP Celloscope equipped with a 76-μm orifice. The Celloscope is operated in a laminar flow hood to minimize particulate contamination. Volume calibrations are carried out using poly (butadiene) latices of known dimensions. The procedures involved are standardized and mononuclear cell populations are known to present no special difficulties.

The volume distribution is of interest because centrifugation procedures which separate on the basis of sedimentation velocity--which in turn is a fairly strong function of cell size and geometry--could be applied if null cells prove to have a different volume distribution from T cells (Pretlow, Th.G.II *et al.*, *Int. Rev. Path.* 9:91, 1975). Since the nylon column effluent suspension will be about 75% T cells the size distribution of this population will largely reflect this cell type.

3.1.2.3.2 Cell Density Distribution

The relative density distribution of the nylon wool column effluent suspension will be measured using gradients of poly (vinylpyrrolidone)-coated silica gel

(Percoll*) as the gradient forming material (Wolff, D., *Methods in Cell Biol.* 10:5, 1975). The gel gradients are formed spontaneously during centrifugation of the cell/gel mixture. This support medium is extremely well suited to isopycnic cell separations since it has low osmolality (<20 m)s/kg H_2O), low viscosity (10-15 kPa·s[cp]) and is completely non-toxic to cells. Density distribution will be determined relative to normal fresh human erythrocytes according to the procedure of Wolff (Wolff, D., *Methods in Cell Biol.* 10:5, 1975). The optimal conditions of centrifugation speed and time will be determined empirically. Stationary distributions will be unloaded with a hematocrit needle and peristaltic pump into a fraction collector. Fractions will be assayed after dilution by cell counting in the Celloscope, using the size discriminators to eliminate counts due to the gel particles. Parallel distributions will be run on human erythrocytes which will act as internal density standards; spleen cell density distributions will be expressed relative to the fraction containing the highest red cell concentration. Since the isopycnic distribution of cells in the gel gradient is both an analytical and preparative procedure, and since Bach has reported that null cells can be partially separated from T cells on relatively crude discontinuous albumin gradients (Bach, M.-A *et al., Ann. N.Y. Acad. Sci.* 249:316, 1975) these experiments will be of considerable interest.

3.1.2.3.3 Cell Electrophoretic Mobility Distributions

The electrophoretic mobility distributions of column effluent cells will be determined using a cylindrical chamber microscope electrophoresis apparatus equipped with Ag/AgCl electrodes as described by Seaman (Seaman, G.V.F. in "The Red Blood Cell," Vol. 2, p. 1136, D. Surgenor (ed.), Academic Press, New York, 1975). The individual velocities of at least 50 cells will be determined by timing their migration at the stationary level in the chamber using a Hewlett-Packard 5304A Timer/Counter interfaced on-line with a Hewlett-Packard 9815A Calculator and 9872A Plotter. The output obtained is a hard copy of the frequency histogram of the mobility distribution of the population with accompanying descriptive statistics such as mean mobility, standard deviation, skewness,

*Percoll is a registered trademark of Pharmacia, Inc.

etc. Mobilities determined in this way are absolute descriptives which do not depend on the properties of calibration particles.

For the mobility distribution determinations cells will be suspended in the low ionic strength buffer of Boehmer (Boehmer, H.V. *J. Immunol.* 112:70, 1974) which has proven to be an adequate support medium for the electrophoretic separation of mouse spleen cells using CPE. Mobility determinations will also be made on human erythrocytes (to check apparatus operation) and column effluent cells suspended in physiological saline to allow data comparison with literature values. It is the low ionic strength mobilities, which are higher than those obtained at physiological ionic strengths (see Equation (2)), which are of relevance for determining the electrophoretic separability of the populations of interest, however. Analytical cell electrophoresis has been carried out routinely for many years in the Department of Pathology at U.B.C. (Vasser, P.S., G.V.F. Seaman, and D.E. Brooks, Proc. 7th Can. Cancer Res. Conf., Honey Harbor, Ont., 10:268, 1967) and we anticipate no difficulties in its application to the present system.

3.2.3.4.5 Partition Coefficients in Selected Two-Phase Aqueous Polymer Systems

To obtain an idea of the surface character of the column effluent cells and to identify those phase systems which will produce the best separations on CCD, a number of single tube partition experiments will be carried out. Partition experiments are carried out in a thin layer plate containing 20 wells and is split horizontally along its midline to allow the top halves of the wells to be isolated from the bottom halves by displacing by one well diameter half relative to the other. Multiple samples can thus be introduced with the wells in register as mixed suspensions, the phases allowed to settle, then the upp. phase isolated from the interface plus lower phase by displacing the top half plate. The top phase is then sampled and the cell concentration determined by cell counting on the Celloscope. Knowledge of the well volume and total number of cells added allows the partition coefficient to be calculated. In general one looks for phase systems of each type which produces partition of 50% of the cells into the top phase, as this is the condition which will

produce the best distribution after CCD (King, T.P. and L.C. Craig, "Countercurrent distributions," in *Methods of Biochemical Analysis,* D. Glick (ed.), Wiley, NY, 1962).

All phase systems employed will be based on mixtures of dextran and poly(ethylene glycol) (PEG) as these systems are the best understood and have proven their value in other cell separation problems (Walter, H. in *Methods in Cell Biology,* Vol. 9, p. 25, D.M. Prescott (ed.), Academic Press, NY, 19[illegible]). Systems which separate primarily on the basis of charge will consist of 5% dextran 500 ($\bar{M}_w \simeq 500{,}000$) and 4% PEG ($\bar{M}_w \simeq 6{,}000$) (5:4 system) plus mixtures of 0.154 M NaCl and isotonic phosphate buffer, pH 7.4, with the phosphate predominating. Varying the phosphate/chloride ratio varies the potential difference between the phases which in turn varies the partition behavior of the charged cells and allows a system with the desired partition coefficient to be found.

Non-charge-based systems will be of two kinds: dextran/PEG/NaCl mixtures close to the critical point (the critical concentrations of polymers below which phase separation does not occur) and dextran/PEG/NaCl systems to which low concentrations of fatty acid esters of PEG have been added as lipid affinity ligands. By making chloride, which partitions equally between the phases, the predominant anion in the dextran/PEG system, no electrostatic phase potential develops. Cell partition is then determined principally by the degree to which one of the polymers dominates in its interaction with the cell surface. The effect of this cell-polymer interaction can be maximized by working near the critical point for phase separation, where the interfacial tension between the phases is very low. More nonspecific adsorption of cells occurring at the interface, the higher the tension; such disturbing effects are minimized near the critical point. We have shown in dextran/PEG systems near the critical point that the PEG-cell interaction is dominant and that this interaction appears to depend strongly on the characteristics of the lipid portion of the membrane (Walter, H., E.J. Krob, and D.E. Brooks, *Biochemistry* 15:2959, 1976). The effect of this interaction and hence the partition coefficient can be varied by slightly varying the polymer concentrations to move the system nearer to or farther from the critical point.

The lipid affinity systems are run fairly far from the critical point in an uncharged chloride system so that interaction with the polymer-ligand will draw the cells up into the top, PEG-rich phase. The partition coefficient is manipulated by varying the concentration of ester in the system at a constant polymer concentration (typically 5:4). All of the above experiments will be carried out at both 4°C and room temperature to further characterize the system behavior.

3.1.2.4 Characterization of Nu/Nu Spleen Cells After Passage Through a Nylon Wool Column

In order to select the optimal procedures and conditions for the isolation of null cells from C3H spleens, information regarding the physicochemical characteristics of these cells is required. An available analog to C3H null cells is, obviously, the cell population obtained from nude mouse spleens after passage through nylon wool columns. Spleen cells for this work will be from "associate flora" nu/nu mice housed under gnotobiotic conditions. This population will therefore be characterized by the procedures described in 3.1.2.3.1 to 3.1.2.3.4 and the relevant parameters compared to those of the B cell-depleted C3H spleen cell population. Comparison of the electrophoretic mobility distributions and partition coefficients will be of particular interest since these are the parameters which would seem *a priori* to be the most likely to differ between the two populations and might therefore be expected to provide the basis for the separation required.

Spleen cells from nu/nu mice will be frozen in Houston by the procedure developed in Task 1 and shipped to Vancouver. It is because the nude mouse colony is located in Houston, necessitating the shipment of cells to Vancouver for characterization, that the C3H cells will be frozen and transported in parallel fashion. This treatment should control against the detection of unique artifactual characteristics in the nude spleen cell population induced by their freezing and transportation. Upon receipt the cells will be revived by the standard procedure and incubated on nylon wool columns. The B-depleted population will then be characterized with respect to cell size, density, and electrophoretic mobility distribution and by their partition behavior in selected phase systems exactly as described for C3H spleen cells. The only difference

anticipated in dealing with B-depleted nude spleen cells as opposed to the equivalent C3H population is that there will be fewer cells per spleen with which to work in the former case. Sufficient nude spleen populations can be shipped to make up for this deficiency, however,

3.1.3 Task 3--Determination of the Minimum Concentration of Null Cells and the Maximum T Cell Contamination which Permits Reliable Thymic Hormone Bioassay

This determination is of importance to avoid overspecification of system requirements. Obviously, the smaller the number of null cells required and the greater the T cell contamination permitted, the less rigorous must be design of a separation system. Indeed, if the requirements are sufficiently flexible it may be possible to achieve adequate separation on earth.

In these experiments, indicator cell preparation will be prepared from the spleens of germ-free nude mice. To these cells will be added 1%, 5%, 10%, 15%, 20%, and 25% C3H mouse thymocytes. An induction experiment identical to that is described in Section 3.1.1.4.

3.1.4 Task 4--On the Basis of Experimental Data Select a Procedure(s) for the Isolation of C3H Null Cells which are Theoretically Most Suitable for Further Experimental Development

At the completion of Task 3, sufficient experimental evidence should be on hand to point to a method(s) offering the promise to achieve the desired objective--the harvest of null cells from immunologically competent C3H mice. Sources other than C3H mice are considered unsuitable, as previously discussed (paragraph 2.4.3).

Alternatively, it is possible that there will be no clear indication that any of the procedures investigated will prove promising.

An Interim Report will be prepared at this time. The report will summarize the findings of Phase I and will present conclusions and recommendations for proceeding to Phase II.

3.2 Phase II

During Phase II, we will build on the firm foundation of experimental data developed during Phase I to actually attempt isolation of null cells from the spleens of C3H mice. It is obviously not possible at this time to predict just what these approaches will be, but based on the rationale presented earlier, we feel there are three general approaches which may prove feasible. These will be rigorously investigated as described in the following tasks.

3.2.1 Task 1--Attempt Elimination of T Cells from C3H Spleen Cells by Techniques Based on the Immunologic Reactivity of T Cells

3.2.1.1 Treatment of Column Effluent Population with Anti-Thy 1.2 Plus Complement Then Protease to Attempt Solubilization of Interferring T Cells

Anti-Thy 1.2 antigen and complement is made by immunizing Thy 1.1^+ AKR mice with thymocytes from Thy 1.2^+ C3H mice with repeated immunizations over a 6-8 week period (Reif and Allen, *J. Exp. Med.* 120:413, 1964). Guinea pig complement (GIBCO) is used. Both the antiserum and complement is used at optimal titers as determined with thymocytes from C3H mice. The cytotoxicity incubation is as described (Twomey *et al., PNAS* 74:2541, 1977). This involves one hour of incubation is a tissue culture incubator with antibody plus complement with a paired incubation using a decomplemented complement source. The purpose of incubation with protease is to stabilize injured T cells (Stewart and Goldstein, *J. Lab. Clin. Med.* 84:425, 1974).

Several potential problems exist with this procedure. Protease treatment may interfere with subsequent null cell-hormone interaction by digesting all or part of the null cell-hormone receptor. Also, released intracellular components may prove to be a problem.

3.2.1.2 E-Rosette Formation Followed by Centrifugation

T lymphocytes adhere to sheep erythrocytes to form rosettes. These rosettes will be prepared using the method of Bach *et al., Ann. NY Acad. Sci.* 249:186, 1975). After rosette formation, the cells will be layered on top of a Ficoll-Hypaque mixture and centrifuged so that rosetted lymphocytes will be

sedimented (Boyum, *Scand. J. Clin. Lab. Invest. Suppl.* 97:77, 1968). The cells that remain above the Ficoll-Hypaque mixture will therefore be depleted of rosetting T cells and will be used in subsequent evaluation.

If either of the procedures attempted in Task 1 proves effective, there will be no apparent reason for going to space and corresponding recommendations will be made.

3.2.2 Task 2--Separation of C3H Spleen Null Cells from T Cells by Countercurrent Distribution of Effluent Cells from Nylon Wool Columns in Two-Phase Aqueous Polymer Systems

The characterization of B-depleted C3H and nu/nu spleen cell populations with respect to their partition coefficients in charge-dependent and non-charge dependent phase systems, as well as in phase systems containing lipid affinity ligands, will dictate the choice of phase composition employed in CCD runs. In general, systems in which the mean partition coefficients of the null and other C3H subpopulations are most different will be used. C3H mice will be maintained in the animal holding facility of the Department of Pathology at the University of British Columbia. Spleen cells will be freshly prepared and depleted of B cells for each experiment.

CCD runs will be carried out as described by Walter (Walter, H., in "Methods in Cell Biology," Vol. 9, p. 25, D.M. Prescott (ed.), Academic Press, NY, 1975) using a thin-layer 120 transfer apparatus manufactured by IRD, Bromma, Sweden. The apparatus is designed for use with polymer phases. Settling times per transfer are dictated by the phase composition but are generally of the order of 5-6 minutes at 4°C, and less at room temperature. Run times will depend on the number of transfers required to effect separation; frequently two 60-step runs are made simultaneously on a 120-well CCD plate with a total run time of 5 hours. Analysis of the distribution curves will be carried out on diluted aliquots from every 5th or 10th cavity using the Celloscope to obtain cell counts. Fractions containing separated peaks or shoulders will be pooled, washed out of the phase system into the cryoprotective medium, and frozen according to the protocol developed in Phase I for shipment from Vancouver

to Houston. The isolated fractions will then be tested for null cell content using the thymopoietin bioassay and the success of the separation determined with reference to the results of Phase I, Task 3. Appropriate controls of unseparated B-depleted C3H spleen cells will be frozen and sent to Houston for bioassay with each set of samples.

The sample sizes involved will be dictated by the degree of separation found in early runs but no difficulty is anticipated in this area based on the known cell content of C3H spleens and our previous experience with CCD separations of mononuclear cells. If cell viability proves to be a problem, the phase system can be enriched with minimum essential medium (MEM) and the high molecular weight fraction obtained by Amicon filtration of fetal calf serum (FCS) against MEM. Complete FCS is to be avoided because of its likely content of thymic hormone and heteroantibody to mouse cells. All solutions in contact with cells will contain antibiotic to minimize bacterial growth.

If CCD on unmodified B-depleted C3H spleen cells does not provide an adequate isolation of null cells, it may well be possible to carry out the separation after exposure of the cells to anti-Thy 1.2 serum with or without complement. Moreover, reaction with the antiserum is likely to lower the surface charge density of the T cells (Coulter, C., *J. Gen. Physiol.* 3:309, 1921). Cell death, as induced by complement fixation, is frequently found to be accompanied by a dramatic decrease in partition coefficient in charge-dependent phase systems (Walter, H., personal communication). These should be a promising approach to the separation of null cells since this cell type should not be affected by the antiserum.

If this latter approach becomes necessary, the antiserum available in Houston will be utilized under the conditions studied in Task 3.2.1.1. CCD will be performed after incubation with anti-Thy 1.2, with control separations from the same cell population not exposed to the serum run on the same CCD plate. Cells will be washed free of excess antiserum before CCD. The resulting distribution and degree of null cell isolation will then be determined as above.

3.2.3 Task 3--Separation of C3H Spleen Null Cells from T Cells by Continuous Flow Electrophoresis of Effluents from Nylon Wool Columns

Separation of C3H null from T cells will be attempted using the Beckman Mark II Continuous Particle Electrophoresis (CPE) System. This features cross-section illumination and a dual-field chamber with total effective field length of 60 cm adapted for paripotential focusing (Strickler, A. and T. Sacks, *Annals N.Y. Acad. Sci., 209,* 497, 1973). The instrument represents the current state of the art in respect to resolution, flexibility, and general convenience of use.

The sample for CPE will be freshly prepared from a spleen cell suspension depleted of B cells and macrophages by passage through nylon wool. The mixed null and T cell samples will be run with and without treatment with anti-Thy 1.2 antibody plus and minus complement.

The media used for cell maintenance during tissue extraction, filtration, freezing, and antibody treatment are of relatively high electrical conductivity. Before separation in the CPE, the cells (treated or untreated with antibody/complement) must be resuspended in lower-conductivity CPE buffer. This can be done by several washing and centrifugation steps, with an intermediate normal saline wash if necessary. Appropriate buffer compositions have been developed and used by other investigators (Zeiller, K. and K. Hannig, Hoppe-Seyler,s *Z. Physiol. Chem.* 352:1162, 1971, and Boehmer, H.V., *J. Innunol.* 112:70, 1974).

Trial runs on the CPE will confirm the general mobility and deflection range of the cellular components of interest. This will help in the selection of appropriate gel coatings for the electrophoresis chamber walls. In conjunction with paripotential focusing, the coatings assure optimum resolution at any given sample flow rate. The trial runs will also establish optimum operating values for electric field strength, chamber buffer flow, and sample flow.

In the separation experiments, fractions will be collected, centrifuged, resuspended, and rate-frozen in a cryoprotective medium. They will then be shipped to Houston and assayed to determine effectiveness of electrophoretic

separation with respect to null cell isolation. If early assays indicate that resolution is inadequate, operating conditions will be modified, using reduced sample processing rates or lower conductivity buffer if necessary.

3.2.4 Selection of Optimal Purification Method and Consideration of Space Advantages

Phase II will conclude with the selection of the procedure considered to be most suitable for the separation of null cells from C3H mouse spleens. It may be, at the conclusion of these studies, that adequate techniques for use on earth will have been developed. However, it is equally possible that use of the selected technique in space will offer significant advantages. Indeed, use in space may make the difference between a feasible separation and an unfeasible separation. At this point in time, we have no way of knowing. Both the CCD and CPE processes can, in principle, be carried out more effectively in a microgravity environment.

If a recommendation for going to space is made, however, this recommendation will be based upon data obtained from rigorously controlled investigations and the rationale for going to space will be obvious. Decisions to proceed to subsequent phases, e.g., development of flight hardware, can be made objectively.

4.0 SCHEDULE

The program described in this report will require 18 months to perform (Figure 4-1). Phase I will be completed in nine months and will include the submission of an Interim Report. Phase II will also require nine months; a second Interim Report will be submitted.

PHASE	ITEM/TASK DESCRIPTION
I	Task 1--Freezing and Storage
	Task 2--Characterize Cell Properties
	C3H • Cell Size Distribution
	• Cell Density Distribution
	• Cell Electrophoretic Mobility Distribution
	• Partition Coefficients
	Nu/Nu • Cell Size Distribution
	• Cell Density Distribution
	• Cell Electrophoretic Mobility Distribution
	• Partition Coefficients
	• Study Antigen Thy 1.2 + Complement Distribution
	Task 3--Detecting Minimum Null/Maximum T Cell Distribution
	Task 4--Prepare Interim Report
II	Task 1--Detecting Immunological Reactivity T Cell
	• Antigen Thy 1.2 + Complement + Protease
	• E-Rosette Formation-Density Gradient Centrifugation
	Task 2--Perform CCD Separations
	Task 3--Perform CPE Separations
	Task 4--Prepare Interim Report, Conclusions and Recommendations

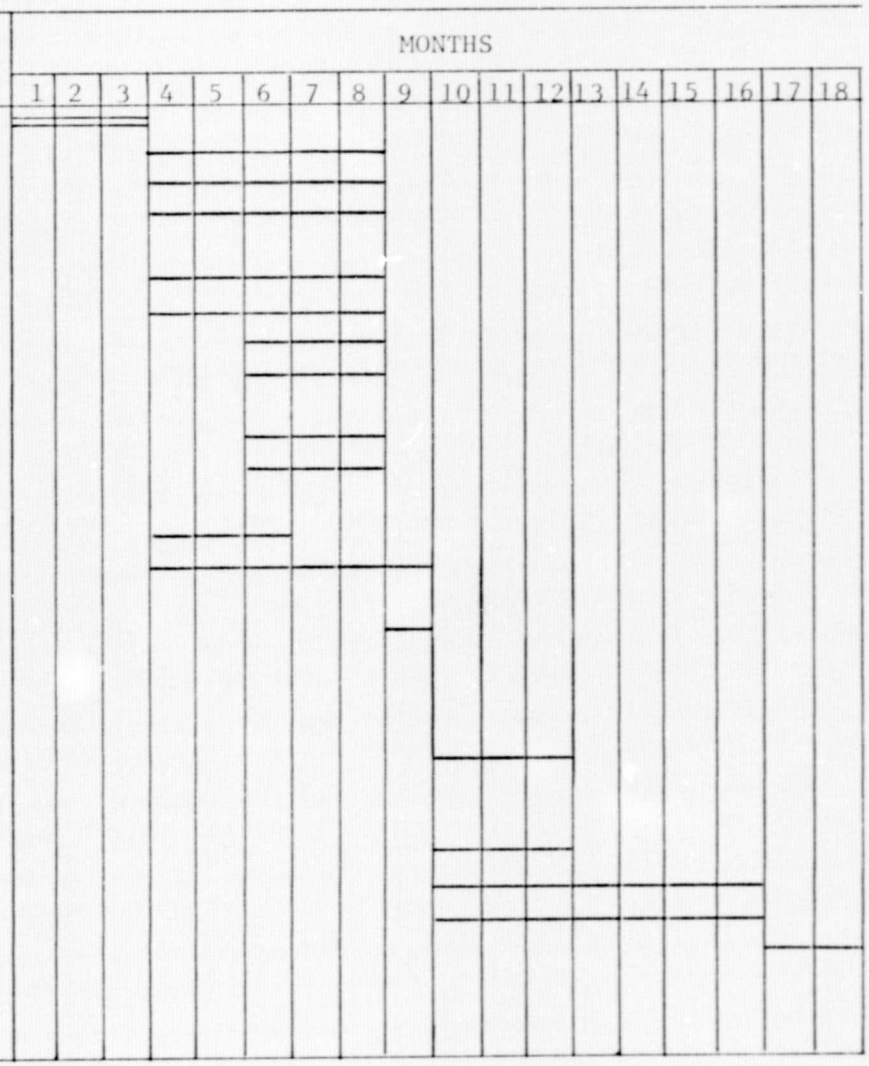

Figure 4-1. Bioprocessing Feasibility Program Schedule

5.0 PROGRAM ORGANIZATION

The organization required to implement the Program Plan is shown in Figure 5-1, below.

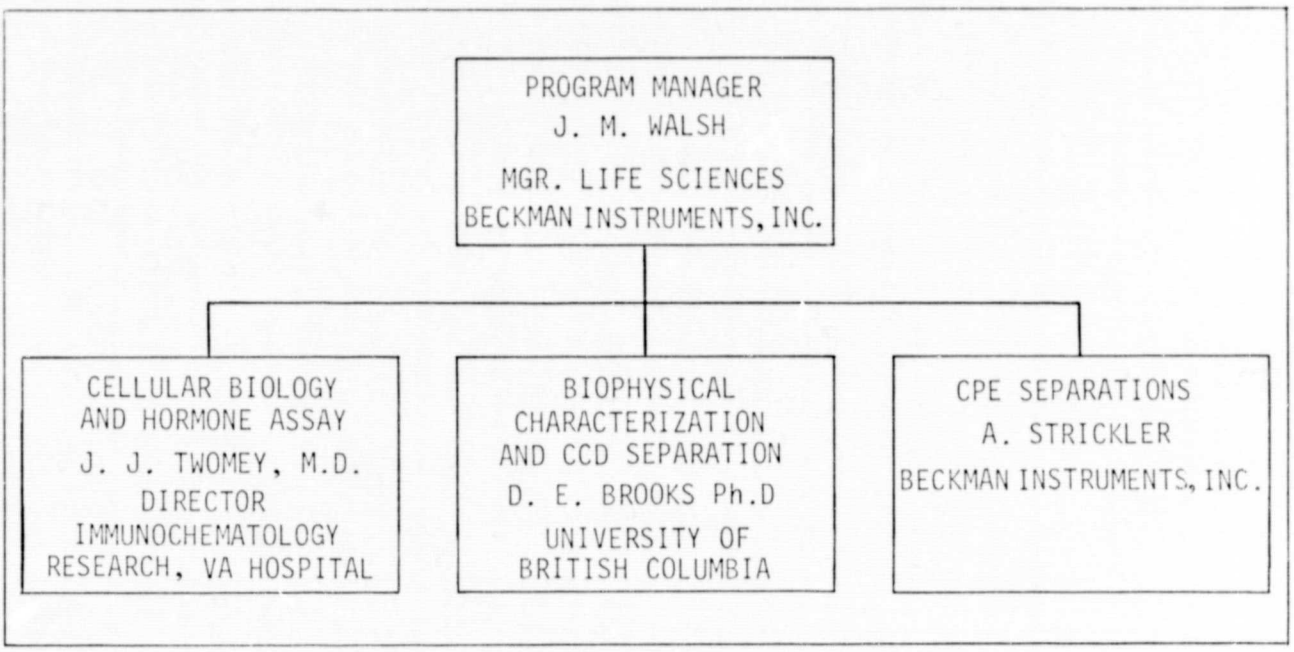

Figure 5-1. Program Plan